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Microbial DNA extraction from samples of varied origin

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The impact of four different soil DNA extraction methods on the quantity and quality of isolated community DNA was evaluated using agarose gel electrophoresis, DNA spectrum study and PCR-based 16S ribosomal DNA analysis. The modified direct lysis method was optimized for environmental water samples (wastewater fed-fisheries and raw liquid sewage canal) as well as diagnostic samples like urine from humans, thus opening up a new arena for fast culture-independent detection of causative pathogens in pathological manifestations like urinary tract infections. The same method was independently found to be effective for isolation of genomic DNA from both Gram-positive and Gram-negative bacteria. Thus a uniform method of DNA extraction from environmental samples (different types of soil and water), pathological samples as well as varied kinds of bacteria was obtained.

Keywords: Community DNA, DNA extraction, microbial biodiversity, urine microbial analysis.

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THE microbial diversity studies conducted in complex ecosystems, such as soil and sewage have often been found to be biased. Essentially this has been due to the inability to culture many microorganisms[1,2]. Reports indicate that only 1 to 4% of the microbes can be cultivated under the standard laboratory conditions[3,4]. In the past decade, applications of new methods based primarily on soil-extracted nucleic acids have provided an alternative to classical culture based microbiological methods, providing unique insight into the composition richness and structure of microbial communities[5]. In case of molecular analysis of microbial communities, the bottleneck remains the isolation of pure community DNA in reasonable quantity. Steps for removing contamination of humic acid and fulvic acid are both complicated and expensive[6]. No universal method for extraction of community DNA from samples of varied origin is available. In the past decade comparative studies have been performed to analyse the efficiency of methods for extraction and purification of soil DNA, revealing that these methods suffer from low efficiency, mainly due to incomplete cell lysis and DNA adsorption to soil particles[7,8]. However, the impact of the extraction method on the outcome of indigenous microbial community analysis has not been clearly established[8]. In recent years DNA analysis has been extensively used for taxonomy as well as diagnostics[9]. PCR-based microbial detection of pathogens in case of meningitis, septicemia, tuberculosis and genitourinary tract infection has already been reported[10–14]. Thus obtaining a viable method for community DNA extraction from samples of varied origin (with minor modifications) and characteristics has become an essential requirement for these studies. This study shows how the modified direct lysis method can actually give good yield of DNA from a large number of varied sources for doing molecular biology work.

Four different methods of DNA extraction[15] with minor modifications were tried with soil samples. The direct extraction method was similar to extraction by bead beating, except that the cell lysis was obtained by repeated inversion of the suspended sample in extraction buffer for 10 min at room temperatures. In each case, 8 ml of extraction buffer was used for 4 g of soil. DNA extractions by sonication as well as enzymatic lysis were followed according to the method of Yeates *et al.*[15]. A modified indirect method of DNA isolation and purification was followed[16].

For DNA extraction from water samples like raw sewage canal and wastewater fed fisheries, the direct extraction method with modification was used. Here the water was centrifuged to pellet down the cells at 10,000 *g* for 10 min and washed thrice with buffer A [50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)] before lysis. For isolation of DNA from urine, the direct extraction method was used with further modifications as mentioned below. The urine was centrifuged at 10,000 *g* for 10 min. The supernatant was discarded and the pellet was washed with phosphate buffer saline before proceeding for lysis. This is to ensure

Table 1. Physico-chemical analysis of soil from different sites of East Calcutta Wetlands. Organic carbon below 0.50 is low, 0.50 to 0.75 is medium and above 0.75 is high. Electrical conductivity is normal below 1.0 mmol cm^{-1}. Soils from all the sites have neutral pH (6.7 to 7.3). The last column represents the amount of DNA (μg) extracted per gram of soil by direct lysis method

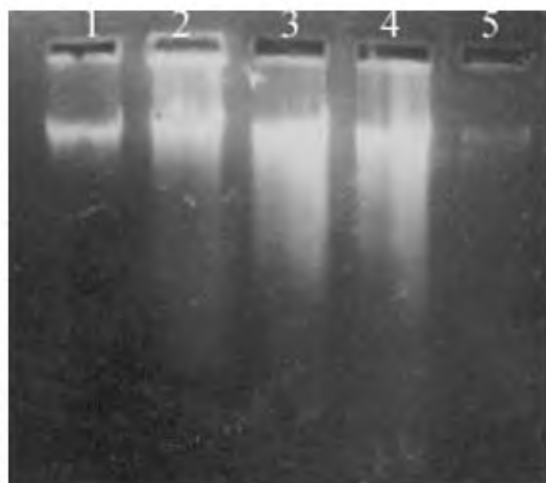|	Mechanical analysis				Per cent organic carbon	Per cent moisture content	EC	DNA μg/g soil
Soil site	Sand	Silt	Clay	Texture				
a	67.2	24	8.8	Sandy loam	0.61	22.31	0.5	6–9
b	71.2	24	4.8	Sandy loam	0.61	22.41	0.5	7–9
c	93.2	2	4.8	Sand	0.54	23.54	0.3	10–12
d	81.2	12	6.8	Loamy sand	0.82	18.53	0.6	16–19

Figure 1. Ethidium bromide-stained 1.5% agarose gel showing community DNA isolated from soil using different isolation methods. Lanes from left to right are as follows: Lane 1, λ DNA (marker); lane 2, Isolation by direct lysis; lane 3, Isolation by sonication; lane 4, Isolation by enzymatic lysis and lane 5, Isolation by indirect lysis.

Figure 2. Photograph of ethidium bromide-stained 1% agarose gel showing lanes loaded from left to right as follows: Lane 1, Marker λ DNA; lane 3, Genomic DNA from novel Gram-negative bacteria; lane 4, Genomic DNA from novel Gram-positive bacteria; lanes 5–15, 16SrDNA PCR product from community DNA of site-b soil (5), site-a soil (6), raw sewage canal water (7), novel Gram-negative bacteria (8), novel Gram-positive protease-secreting strain (9), novel Gram-negative oil-degrading bacteria (10), site-b soil (11), water from wastewater-fed fisheries (12), site-c soil (13), site-a soil (14) and site d-soil (15), and lane 16, 100 bp ladder (Promega).

the removal of loosely attached materials as well as salts from the sample. During lysis, along with lysis buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH-8.0), 1.5 M NaCl), 50 μg/ml proteinase K was added. The direct method of extraction was applied to soils of varied characteristics (Table 1). This method was also applied for extraction of genomic DNA from Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, uncharacterized novel strains) as well as Gram-negative bacteria (*Escherichia coli*, uncharacterized novel strains; Figures 1 and 2).

Co-extracted humic acid is the major contaminant when DNA is extracted from the environmental sample. These compounds absorb at 230 nm, whereas DNA absorbs at 260 nm and protein at 280 nm. To evaluate the purity of the extracted DNA, absorbance ratio at 260/230 nm (DNA/humic acid) and 260/280 nm (DNA/protein) was determined (Table 2, Figure 3). The isolated DNA was analysed on 1.5% agarose gel (Figures 1 and 2).

DNA isolated by the direct method (soil, water, urine), sonication and enzymatic lysis was used directly for PCR amplification, but that isolated from indirect method was used in 1 : 50 and 1 : 100 dilutions for PCR. The reason

for this variation was the presence of higher concentration of co-eluted inhibitors in the later preparation compared to the earlier ones. The concentration of template is adjusted so that the inhibitor concentration is below the critical level and does not inhibit amplification anymore. PCR was performed in a Genecycler (BioRad). Degenerate Universal primers 5′-TGA CTG ACT GAG TGC CAG CMG CCG CGG-3′ and 5′-TGA CTG ACT GAG AGC TCT ACC TTG TTA CGM YTT-3′, M = A/C, Y = C/T (Isogen Life Science, Holland) were used for amplification of the 16 SrDNA fragment (1050 nt) using touch-up program mentioned elsewhere[16]. Here since the primers are degenerate, during PCR the annealing temperature was increased with increasing number of cycles to ensure a stringent amplification at the beginning to remove specific
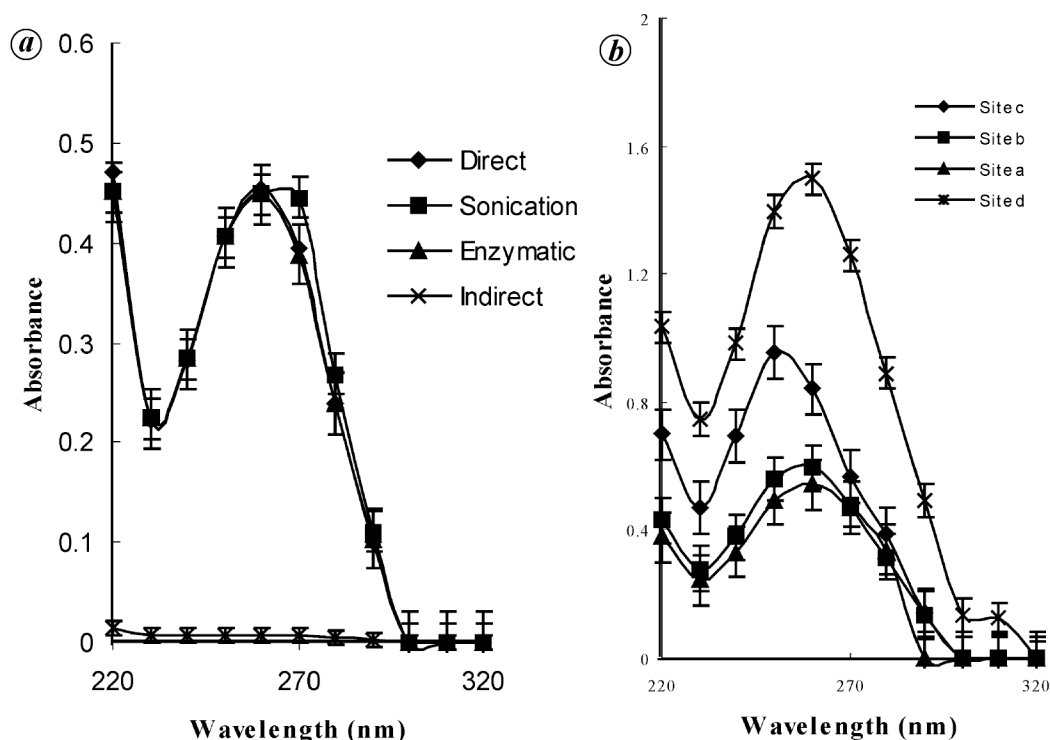
Figure 3. Spectrum of community DNA isolated by four different methods (*a*), and from soils of different sites (*b*).

Table 2. Comparative data of genomic DNA isolated by modified direct lysis, lysis by sonication, enzymatic lysis and indirect lysis from loamy sand. It represents the total yield, A260/A230, A260/A280 ratio. A260/A230 > 2 indicates pure DNA with low organic contamination, 1.7 < A260/A280 ≥ 2 indicates pure DNA

Method	A260/A230	A260/A280	Yield µg/g soil
Direct lysis	2.04	1.895	17
Lysis by sonication	2.05	1.893	19
Enzymatic lysis	2.01	1.88	16.7
Indirect lysis	1.05	2.117	199

products as well as to allow most of the primer pairs to amplify at their respective annealing temperatures subsequently. The reaction mixture (50 µl) consisted of 1–6 µl template (up to 500 ng depending upon the concentration of co-eluted inhibitor), 5 µl of 10 × PCR Buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin) (Bangalore Genei, India), 250 µM deoxyribonucleotide triphosphate (Sigma), 3U Taq polymerase (Bangalore Genei) 0.7 µM of each primer (Isogen Life Science, Holland). The PCR products were analysed on a 1.5% agarose gel (Bangalore Genei) (Figures 2 and 4).

The PCR products were subsequently cloned (where required) and sequenced using the DBT sponsored DNA sequencing facility at Delhi University South Campus (ABI). The GenBank accession numbers of sequences amplified from environmental samples are DQ256262-DQ256264, DQ270005-DQ270006, AY897549-AY897554.
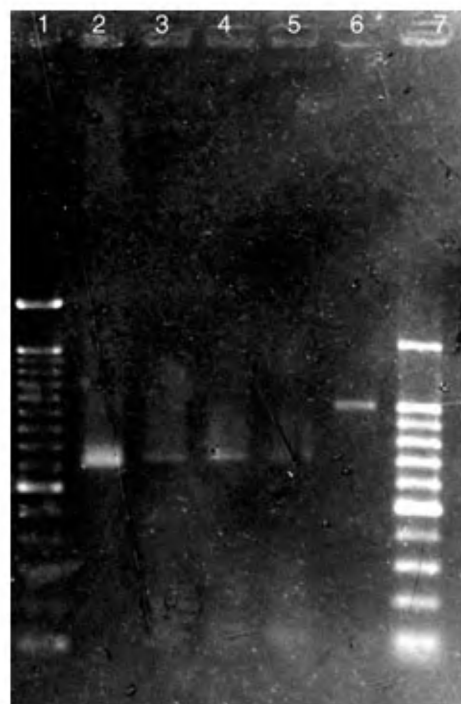

Figure 4. Photograph of ethidium bromide-stained 1.5% agarose gel showing the PCR product obtained from community DNA. Lanes from left to right are as follows: Lane 1: 100 bp ladder (Fermentus); lanes 2–5, Partial 16 SrDNA from urine community DNA (~ 750 bp); lane 6, Partial 16 SrDNA from *E. coli* and lane 7, 100 bp ladder (Promega).

Community DNA was isolated by four different methods. DNA was analysed by agarose gel electrophoresis

(Figures 1 and 2) and UV spectrum analysis (Table 2, Figure 3). The experiments were repeated five times. The results mentioned above show that except from indirect lysis, all other DNA isolates, viz. by direct lysis, lysis by sonication as well as enzymatic lysis were pure and in good quantity. DNA by direct lysis was accepted as the method of choice for further studies (Figure 1) as it gave maximum amount of pure intact DNA (no DNA smear of lower molecular weight). DNA isolation was carried out from soils of different mechanical properties (Table 1) by the direct lysis method (Figure 3 b). Pure DNA was obtained in sufficient quantity (Table 1). The same method when applied to urine; enriched as well as pure cultures of both Gram-positive and Gram-negative bacteria (Figure 2), gave pure DNA which was subsequently used for PCR amplification of 16 SrDNA gene (Figures 2 and 4), TA-cloning (for community DNA and mixed culture) and sequencing. The novel sequences were submitted to GenBank. The applicability of DNA-based methods in the detection of pathogens would minimize the use of only culture-based detection. This becomes essential, as only 1 to 4% of the microbes can be cultivated under standard laboratory condition. Thus here we report one method of DNA isolation which works for a wide variety of samples ranging from soil of different kinds, water bodies with different organic and metal content (data not shown), and pure bacterial cultures of both Gram natures to pathological specimens.

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Agrobacterium-mediated genetic transformation of Nagpur mandarin (Citrus reticulata Blanco)

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Genetic transformation protocol was developed for Nagpur mandarin (*Citrus reticulata* Blanco), a choicest citrus variety grown in India and South East Asia. Cotyledon segments from mature seeds were co-cultivated with *Agrobacterium tumefaciens* for two days and cultured on an adventitive embryo induction

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